The catalytic mechanism of DNA polymerases has been shown to involve multiple steps that precede and follow the transfer of a nucleotide to the 3′-hydroxyl of the growing DNA chain. Here we report a single-molecule approach to monitor the movement of E. coli DNA polymerase I (Klenow fragment) on a DNA template during DNA synthesis with single base-pair resolution. As each nucleotide is incorporated, the single-molecule Förster resonance energy transfer (smFRET) intensity drops in discrete steps to values consistent with single nucleotide additions to the primer terminus. Purines and pyrimidines are incorporated with comparable rates. When a mismatched primer-template junction is used, smFRET is observed consistent with the primer moving into the exonuclease domain. This analysis was used to determine the fraction of primer-termini bound to the exonuclease and polymerase sites. Most interestingly, we observe a structural change following the incorporation of a correctly-paired nucleotide, consistent with transient movement of the polymerase past the preinsertion site or a conformational change in the polymerase. This may represent a previously unobserved step in the mechanism of DNA synthesis that could be part of the proofreading process.

2262-Plat
Single-DNA Detection of Stepwise DNA Compaction by Cohesin SMC Protein Complexes
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Structural Maintenance of Chromosomes (SMC) protein complexes are essential for the precise folding and segregation of chromosomes during cell proliferation in both prokaryotes and eukaryotes. Cohesin SMC complexes are believed to hold sister chromatids together during chromosome segregation. However, how cohesins interact with DNA at the molecular level is still poorly understood. In our current study, we report that yeast cohesin SMC heterodimers condense single-DNA molecules by distinct steps in a force-dependent manner in the absence of ATP. The rate of condensation is supercoiling-dependent, and positive supercoiling of the DNA accelerates the condensation. We also observed stepwise DNA condensation by cohesin SMC complexes (with Scc1 kleisin subunits). Moreover, the DNA compaction by cohesin complexes is regulated by ATP, and requires the torsional rigidity of dsDNA. Our results suggest that cohesins may play a broader role as organizers of chromatin, possibly by defining ‘‘cross-linkers’’ or ‘‘loops’’ through the whole cell cycle.

2263-Plat
Torsional Regulation of hRPA-induced Unwinding of Double Stranded DNA
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Replication Protein A (RPA) is the main eukaryotic single-stranded binding protein and is essential for most aspects of cellular DNA metabolism. RPA is able to unwind the dsDNA helix by binding to transiently forming single-stranded DNA bubbles [1]. We present single-molecule measurements of the dynamics of human RPA (hRPA) activity on dsDNA and ssDNA obtained with a magnetic tweezers-based assay. We show that hRPA-induced dsDNA-helix unwinding is strongly promoted by unwinding torque and can be reversed by rewinding torque exerted on the DNA. Torque thus provides a means for tight mechanical regulation. The torque generated by the application of a modest stretching force (~0.5 pN) on supercoiled DNA is sufficient to overcome the previously reported inhibitory effects of high salt. We propose a torque-based model that explains the dynamics of hRPA duplex (un-)binding and provide mechanistic insight. The mechanochemical regulation of hRPA binding to dsDNA is likely important for replication initiation and for various DNA repair pathways.
We conclude the presentation with single-molecule measurements on the HepA-related protein (HARP), which recently was proposed to act as an annealing helicase that is able to rewind DNA bubbles that are stably bound by RPA [2]. We demonstrate that HARP is able to rapidly re-anneal hRPA-stabilized ssDNA bubbles.
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Platform AS: Exocytosis & Endocytosis

2264-Plat
Localized Plasma Membrane Topological Changes upon Exocytosis Visualized by Polarized-TIRFM
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Total internal reflection fluorescence (TIRF) microscopy images the plasma membrane-cytosol interface and has allowed insights into the behavior of individual secretory granules before and during exocytosis. Much less is known about the dynamics of the other partner in exocytosis, the plasma membrane. Here we report the implementation of a TIRFM-based polarization technique to detect rapid submicron changes in plasma membrane topology as a result of exocytosis. A theoretical analysis of the technique is presented taking into account the high numerical aperture lenses that are used in through-the-lens TIRFM, the point spread function, and pixilation on the CCD camera. Image simulations are presented for predicted topologies of the post-fusion granule membrane/plasma membrane complex. Experimental results on dil-stained bovine adrenal chromaffin cells using polarized TIRFM demonstrate rapid and varied submicron changes in plasma membrane topology at sites of exocytosis. They occur immediately upon fusion in at least 80% of the fusion events and decay with varying speeds from as fast as 100 ms to tens of seconds. Endocytosis, also imaged by optical techniques, revealed that less than 10% of the fusion events stimulated with elevated K^+ result in rapid endocytosis. Thus, most of these topological changes are kinetically distinct from endocytosis and reflect a varying time course for the flattening of fused granule membranes into the plasma membrane.

2265-Plat
Compound Exocytosis in Rat Beta-Cells Triggered by Global Elevation of Cytosolic Calcium
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Exocytosis in β-cells is traditionally thought to involve fusion of individual insulin granules. It has been proposed, however, that granules may prefuse with each other inside the cell and that these aggregates subsequently undergo compound exocytosis. ATP is stored in insulin granules and co-released with insulin. At low $[Ca^{2+}]_i$ (~0.2 micromol/l), most exocytotic events (detected as ATP release in rat beta-cells expressing ATP-sensitive $P2X_2Rs$) were small. However, at 2 micromol/l $[Ca^{2+}]_i$, 20% of the events became 5- to 10-fold larger than those seen at low $[Ca^{2+}]_i$. The small events were associated with capacitance increases of ~3 fF, close to that expected for 300-nm secretory vesicles. Occasionally, we observed much larger stepwise capacitance increases (20-40 fF) indicating that 5-10 secretory granules fused simultaneously. The ATP-induced currents associated with the large capacitance steps were correspondingly increased and rose monotonically (i.e. without signs of superimpositions of several smaller events). This argues that they reflect the emptying of prefused granule aggregates via single fusion pores. Two photon imaging of exocytosis using the fluorescent polar tracer sulforhodamine B (SRB) confirmed that compound exocytosis does not contribute to glucose-stimulated insulin secretion (average diameter: 400 nm). However, in the presence of carbachol (20 micromol/l), exocytosis of large structures corresponding to 4-5 secretory vesicles were observed and accounted for 20% of the events. These large events attained their maximum size within the temporal resolution of the imaging system (0.7 s) and were not slower than the ordinary (small) events. Using 3-dimensional scanning electron microscopy we obtained ultrastructural evidence for the formation of multivesicular structures in beta-cells in islets exposed to carbachol for 5 min. We conclude that compound exocytosis becomes quantitatively significant in response to global elevations of $[Ca^{2+}]_i$ such as those elicited during muscarinic stimulation.

2266-Plat
Exocytosis, Dependent on Calcium Release from Calcium Stores, is Regulated by Calcium Microdomains
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The relationship between the cellular Ca^{2+} signal and exocytic vesicle fusion is a key determinant of the regulation of the kinetics and magnitude of the secretory response. Here, we have investigated secretion in epithelial cells where the exocytic response is controlled by Ca^{2+} release from intracellular Ca^{2+} stores (Ueda & Petersen 1977). Using live-cell 2-photon microscopy, identifying each individual exocytic fusion event (Thorn *et al.,* 2004) and recording cytosolic Ca^{2+} signals with Fura-2 (Nemoto *et al.,* 2001), we show no evidence for spatial clustering of exocytosis; indeed exocytosis is actually specifically excluded from sites of Ca^{2+} release hot-spots. Consistent with this data, loading the cells with EGTA potently blocks exocytosis. These results indicate that the control of exocytosis, triggered by Ca^{2+} release from stores, is through the regulation of cytosolic Ca^{2+} concentrations within large volume microdomains.
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